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UNITED STATES PATENT APPLICATION

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for

PRODUCTION OF TETRAVALENT ANTIBODIES

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PRODUCTION OF TETRAVALENT ANTIBODIES

FIELD OF THE INVENTION

The present invention generally relates to a novel process for the preparation of biologically active antibody dimers and a pharmaceutically acceptable compositions containing such dimers. These dimers can be composed of two antibody molecules having the same antigen binding specificity and linked through a reducible, disulfide, or a non-reducible thioether, bond (homodimers) or, alternatively, can be composed of two different antibody molecules having binding specificity for two distinct antigens (heterodimers). The subject antibody dimers are useful for inducing hyper-cross-linking of membrane antigens. The present invention further relates to the use of biologically active antibody dimers for the preferential killing or inhibition of selected cell populations in the treatment of diseases such as cancer and autoimmune disorders.

BACKGROUND OF THE INVENTION

Monoclonal antibodies were once thought to be an ideal way to target malignant tissues, by delivering a killing agent, while leaving healthy tissue intact.

limitations, bispecific antibodies were developed, which remain bivalent, but are specific for a target cell on one arm of the antibody and a killing agent on the other arm. The killing agent can be a toxin, a drug, a chelated radioisotope, or, more likely, a cytotoxic effector cell.

Monoclonal antibodies can also show therapeutic activity against specific cells, e.g., malignant tissues based on the interaction of the Fc portion of the antibody heavy chain with other components of the immune system, such as the complement cascade or by binding to Fcγ receptors or various cytotoxic effector cell types.

Another means of effecting cell death comprises inducing the cross-linking of membrane antigens. Previous studies have indicated that antibody cross-linking of membrane B-cell markers (e.g., surface IgM, Valentine et al., *Eur. J. Immunol. 22*:3141 (1992); and MHC class II, Newell et al., *PNAS 90*:10459 (1993)) can inhibit malignant B cell proliferation and in many cases induce apoptosis (e.g., programmed cell death) *in vitro*.

Shan et al. (*Blood 91*:1644-1653) demonstrated that hyper-cross-linking of the CD20 antigen, by using the murine 1F5 antibody cross-linked with a goat anti-

linking of membrane bound MAb was amplified with a anti-mouse IgG (Chaouchi et al., *J. Immunol. 154*:3096 (1995)).

It may be possible that hyper cross-linking of these surface membrane markers could augment the existing anti-tumor activities of MAb's like C2B8, a chimeric monoclonal antibody specific for CD20, and increase therapeutic effectiveness. Therefore, molecules that can induce cell death in a pharmaceutically acceptable format would potentially provide an attractive therapeutic agent for immunotherapy of neoplastic disease.

Apparently with that goal in mind, Wolff et al. (*Cancer Res. 53*:2560-2565 (1993)) and Ghetie (*PNAS 94*:7509-7514 (1997)) have reported the chemical synthesis of several IgG/IgG homodimers to carcinoma associated surface antigen (BR96 and HER-2). The Ghetie dimers also included antibodies to several human B-cell markers (CD20, CD19, CD21, CD22). In this approach, one portion of the molecule was functionalized using a linker designed to introduce a reactive thiol on the antibody, while the other Ab portion used a linker to introduce a maleimido group. When purified from unreacted linkers and mixed together, the two antibodies complex by formation of a thioether (non-reducible) bridge that links the

However, unfortunately, the yields of the 300 kDa IgG-homodimer were very low (20-25%) and were similar or lower than "spontaneously" formed CD19 homodimer, which ranged from 20-30% (Ghetie et al., *PNAS 94*:7509-7514 (1997)). Reducing SDS-PAGE gels of purified homodimer showed only a small percentage was linked via a thioether bond, indicating most of the dimers formed using this methodology may have been naturally occurring or mediated through disulfide bridging. Nevertheless, all of the purified dimers were growth inhibitory, although only the anti-carcinoma (Her-2) dimer and not homodimers directed against B cell markers CD19, CD20, CD21, CD22 were reported to be apoptotic. Additionally, the anti-CD19 homodimer was tested in animal models and shown to have anti-tumor activity. However, there is a need in the art for a more efficient method for producing homodimers, in particular for homodimers or heterodimers that are capable of initiating apoptosis, e.g., in proliferating malignant B-cells populations.

In the present invention, two monoclonal antibodies were used: a mouse human chimeric antibody specific for CD20 (C2B8), and a Primatized® antibody specific for CD23 (p5E8). Low grade and aggressive B-cell lymphomas express the B cell antigens CD20 and CD23. CD20 is a non-glycosylated 35 kDa B-cell

Tedder et al., *Immunol. Today 15*:450-54 (1994)). The antigen appears as an early marker of the human B-cell lineage, and is ubiquitously expressed at various antigen densities on both normal and malignant B cells. However, the antigen is absent on stem cells or pre B cell populations, as well as on the fully matured plasma cell, making it a good target for antibody mediated therapy. CD23 is the low affinity receptor for IgE. Antibodies to CD23 have been suggested to be useful for treating allergic and inflammatory responses. In fact, IDEC Pharmaceuticals, Inc., the assignee of this application, has an application pending relating to the use of an anti-CD23 antibody of the IgG1 isotype for therapeutic usage. Of importance herein, CD23 is expressed on B-cells, and particularly by B-cell lymphoma cells.

While only a small fraction of the CD20 antigen is expressed on the surface membrane, MAb's binding to the extracellular domain have had variable activities in promoting or inhibiting B cell function. For example, the anti-CD20 MAb, 1F5, was originally shown to activate resting (G_0) B-cells into (G_1/S/G_2) proliferating populations (Clark et al., *PNAS, USA, 82*:1766-70 (1985)). Additionally, Holder et al. (*Eur. J. Immunol. 25*:3160-64 (1995) demonstrated that Mab 1F5 cross-linking of the CD20 surface antigen protected proliferating tonsular B cells from

Today 15:450 (1994), was not stimulatory for resting B cell populations (Tedder et al., *Eur. J. Immunol. 16*:881 (1986)).

Despite differences in activity using normal B cell populations, murine anti-CD20 MAb's (*e.g.*, 1F5, B1, B20 and 2H7) had no effect on growth inhibition of proliferating human (CD20+) lymphoma cell lines *in vitro*, but *in vivo* showed tumor growth inhibition using human lymphoma mouse xenograft models (Press et al, *Blood 69*:584-591 (1987); Shan et al., *Blood 91*:1644-1653 (1998); Funakoshi et al., *J. Immunol. 19*:93-101 (1996); Hooijberg et al., *Cancer Res. 55*:840-846 (1995); and Ghetie et al., *PNAS 94*:7509-7514 (1997)). The mechanism mediating anti-tumor activity remains unclear but may be mediated through complement dependent cell killing (CDC) or antibody dependent cell killing (ADCC), both of which are dependent on activation of host cell mechanisms through the Fc portion of the MAb after CD20 binding. Indeed, Funakoshi et al. (*J. Immunol. 19*:93-101 (1996)) has shown that the anti-tumor activity of 2H7 *in vivo* was blocked when Fc receptor was blocked or with a $F(ab)_2$ antibody.

The chimeric MAb used in the present invention (C2B8) was developed at IDEC Pharmaceuticals Corporation for treatment of human B cell lymphoma (Reff

Ovary cells. MAb C2B8 maintains the 2B8 murine variable region coupled to the human gamma 1 heavy chain and human κ light chain constant regions. Like its murine counterparts, C2B8 was not growth inhibitory and does not induce apoptosis of human lymphoma cell lines *in vitro*, but does demonstrate anti-tumor activity when tested *in vivo* using murine xenograft animal models.

Chimeric C2B8 efficiently binds human complement, has strong FcR binding, and can efficiently kill human lymphocytes *in vitro* via both complement dependent (CDC) and antibody dependent (ADCC) mechanisms (Reff et al., *Blood 83*:435-445 (1994)). C2B8 was also strongly depleting of B cells in human Phase I/II clinical trials, but was nevertheless shown to be safe and effective with most side effects infusion related (Maloney et al., *Blood 84*:2457-2466 (1994) and Maloney et al., *JCO 15(10)*:3266 (1997)).

The antibody showed an overall response rate of 48% in patients with low grade or follicular lymphoma (McLaughlin et al., *JCO*, in press). However, the response rate decreased dramatically (34%) in chemo-resistant patients who failed to respond to their last chemotherapy regime (McLaughlin et al., *Proc. Am. Soc. Clin. Oncol. 16*:16a (Abstr. 55) (1997)). Additionally, the antibody showed poor

[illegible] Therefore, the need to increase the effectiveness of [illegible]

immunotherapy and, specifically, using C2B8 or CD23 antibody therapy remains a high priority in the treatment of human leukemia and lymphoma patients. The anti-CD23 antibody exemplified in the methods herein was also developed by IDEC and is a primatized anti-CD23 antibody of the IgG1 isotype.

OBJECTS OF THE INVENTION

Based on the foregoing, an object of the invention is to provide novel therapeutic agents, in particular antibody dimers for use in antibody therapies.

More specifically, it is an object of the invention to provide novel antibody dimers having specificity to CD23 and/or CD20 antigen.

It is a more specific object of the invention to provide an efficient method for producing stable antibody dimers, especially IgG/IgG homodimers.

It is another object of the invention to provide novel therapies involving the administration of antibody dimers.

It is a more specific object of the invention to provide novel methods for treating cancer, and autoimmune or allergic disorders by administering antibody dimers.

It is another object of the invention to provide novel therapeutic

allergic disorders, autoimmune disorders

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 contains DNA and predicted amino acid sequences of a "dimeric anti-CD20 light chain (version 1).

Figure 2 contains DNA and predicted amino acid sequences of a "dimeric" anti-CD20 heavy chain (version 1).

Figure 3 is a schematic map of expression construct used to express the subject antibodies.

Figure 4 contains structures of C2B8 (αCD20) homodimer and C2B8/p5E8 heterodimer (αCD20/αCD23).

Figure 5 contains SDS/PAGE results comparing C2B8 (-s-s-) homodimers and C2B8/p5E8 (-s-) heterodimers to starting material.

Figure 6 contains SDS/PAGE results comparing C2B8 (-s-s- and -s-) homodimers and C2B 8/p5E8 (-s-) heterodimers to starting material.

Figure 7 contains HPLC analysis of C2B8 homodimers.

Figure 8 contains HPLC analysis of C2B8/p5E8 heterodimers (αCD20/αCD23 dimer).

Figure 9 shows binding of C2B8 (-s-s-) homodimer to CD20 psotive cell

Figure 10 contains results of a competitive binding assay of C2B8 and C2B8 (-s-s-) homodimer on SKW cells.

Figure 11 shows binding of αCD20/αCD23 heterodimer (C2B8/p5E8) to SKW and DHL-4 cell lines.

Figure 12 shows binding of αCD20 C2B8 homodimer and αCD20/αCD23/p5E8 heterodimer to SKW cells (CD20+/CD23+).

Figure 13 shows anti-tumor activity of C2B8 chemical (-s-s-) dimers on Daudi tumor xenografts.

Figure 14 shows anti-tumor activity of C2B8 (-s-s-) dimers on Daudi tumor xenografts.

Figure 15 shows apoptotic activity of C2B8 (-s-s-) homodimer.

Figure 16 shows apoptotic activity of C2B8/p5E8 (s) heteromer.

Figure 17 shows growth inhibition of B-lymphoma CD20/CD23 positive cell lines (SB and SKW) afer 96 hours continuous exposure to MAb.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The following description will enable a person skilled in the art to which this invention pertains to make and use the invention, and sets forth the best modes

As discussed, the present invention generally relates to a process for the preparation of biologically active antibody dimers and pharmaceutical composition containing such antibody dimers. The present invention further relates to the use of biologically active antibody dimers for the preferential killing or inhibition of selected cell populations in the treatment of diseases such as cancer and autoimmune disorders.

Previously, homodimers were chemically generated from naturally occurring monoclonal antibodies by using chemical cross-linkers to introduce a thioether bond between the two IgG antibodies (Ghetie, *PNAS 94*:7509-7514 (1997)). Because the dimers are formed using chemically functionalized antibodies, one cannot control where the thioether linkage occurs. As a result, the Ghetie method yielded a low amount of homodimers and resulted in a mixture of naturally occurring, disulfide linked homodimers and the chemically generated thioether linked homodimers.

Because of the need for a method which produces an increased yield and chemical purity of IgG homodimers, applicants set out to develop the method of the present invention. The present invention is distinguished from Ghetie by the use of monoclonal antibodies which have had a cysteine residue genetically engineered at

chemically introduce a reactive thiol group.

The method of the present invention increases yield of homodimer formation to 40-50% of the starting material, and is applicable for preparing either disulfide or thioether linked antibody homodimers, preferably IgG/IgG homodimers. Additionally, preparation of thioether linked homodimer was more efficient than the Ghetie method as determined by SDS-PAGE (reducing) gels. Because of the high yield and efficiency of thioether linked homodimers, this method, unlike the Ghetie method, can also be used for preparing antibody heterodimers (preferably IgG/IgG heterodimers), in which each antibody arm is directed against different antigens.

Also, surprisingly and quite unexpectedly, when compared to the Ghetie anti-CD20 dimers using MAb 2H7, the C2B8 dimers using this present method (homodimers and heterodimers) were capable of initiating apoptosis in proliferating malignant B-cell populations. More importantly, these dimers were strongly growth inhibitory for lymphoma cells in culture, showing a 200-fold increase in potency over dimers prepared according to the method of Ghetie. Homodimers (disulfide linked) were also evaluated in animals and shown to have better therapeutic activity than the parent molecule C2B8.

The monoclonal antibodies used for the present invention can be any

from any mammalian host. Although in the examples the Ghetie antibodies

at position 444 of the heavy chain, the location of the cysteine is not limited to this position, and the invention embraces incorporation of cysteine at other sites. In fact, other sites on the antibody may be better suited for cysteine placement. In this regard, the placement of cysteine at position 444 may not be preferred because the cysteine molecule (one on each arm) is close in proximity to the cysteine on the neighboring heavy chain such that an intrachain disulfide bond may form. Therefore, it may be preferable to place cysteine at a different site, e.g., on the outside loop of a domain where the cysteine molecules would physically be further apart. Thereby, the potential for the formation of intrachain disulfide bonds would potentially be eliminated or minimized.

Three specifically contemplated alternative positionings with the anti-CD20 antibody 2B8 could include replacing the serine residue at position 416, the glutamine residue at position 420, or the glycine residue at position 421. These sites have been selected cognizant of the fact that one desires to enhance dimer formation yet retain the antibody affinity and effector functions as much as possible. Also, it is anticipated that other sites may also provide for effective dimer formation.

It is desirable to eliminate intrachain disulfide bonds so that the cysteine thiol

cysteine thiol by maleimides, oxidation of two adjacent thiol groups to a disulfide bond, or through disulfide interchain bonds with pyridyl protected disulfides.

Various molecular biological techniques (including, but not limited to site directed mutagenesis, PCR mutagenesis, random mutagenesis, restriction fragment subcloning, DNA synthesis, etc.) can be employed by one skilled in the art to insert the cysteine at the appropriate site with the resultant antibody molecule. In the examples that follow, site directed mutagenesis was used. Production of the recombinant antibody then, in general, includes introduction of a recombinant gene encoding an antibody heavy chain into any suitable host cell together with a recombinant gene encoding an appropriate antibody light chain. The transfected cells can either be grown *in vitro* or *in vivo*.

As discussed above, placement of the engineered cysteine at position 444 of the heavy chain resulted in intrachain disulfide formation. Therefore, the molecule must be partially reduced before dimerization can proceed. It is anticipated that changing the placement of the introduced cysteine would eliminate this step. However, for these engineered molecules, the disulfide bond (S-S) formed between the neighboring cysteine molecules on the genetically engineered

partially reduce the antibody molecules using dithiothreitol (DTT) or 2-mercaptoethylamine

selectively expose specific thiols. Partial reduction at 37°C requires a range of reducing agent concentration from about 1 to 3 molar excess.

However, these reaction conditions can be modified. For example, the reaction can be effected at lower temperatures or with other reducing agents, such as mercaptoamines or mercaptoethanol. These reaction conditions may require a higher molar excess, which may be readily determined using routine experimentation by one of skill in the art. Under the limiting conditions used, these agents will reduce the most accessible cysteine first. Thus, it is important that the genetically engineered cysteine molecule be positioned correctly and be readily available for reduction. This will increase the likelihood that the genetically engineered cysteine will be the molecule forming bonds with cysteines or other thiol reactive groups on other antibody molecules. Additionally, the introduced cysteine must be positioned correctly on the heavy chain so as to not interfere with FcγR binding or complement activation. This can be determined by trial and error experimentation.

The methods of the present invention produce either dimers formed by disulfide bonds or dimers formed by thioether linkage. In the case of disulfide

[illegible] after reactive is added to the

antibodies which forms a bridge between the two antibody molecules. There are a variety commercially available of maleimido cross-linkers which can be used for the present invention. These cross-linkers bind on one side to a thiol group and on the other side to any of a variety of molecules (for example, lysine, a carboxyl group, etc.) which are naturally present on an antibody molecule. In this way, a dimer can be formed between an antibody which has been modified to contain a cysteine molecule at a specific position and another antibody which has not been modified. By using special conditions (i.e. purifying the selectively reduced MAb by applying it to a PD-10 column and equilibrating with deoxygenated normal saline containing sodium citrate (10mM) and EDTA (1mM)), which discourage the formation of homodimers via a disulfide bond, one can be assured that only dimers formed by a thioether linkage are produced.

Unlike the Ghetie method, which results not only in chemically induced dimers but also naturally occurring dimers, the method of the present invention produces very little if any naturally occurring dimers, and thus obtains a high yield of the desired dimer. The dimers produced by the present invention also, surprisingly, enhanced apoptotic activity of B cells from chronic lymphocytic

used. CLL B cells express low levels of CD20, and previous attempts to activate complement mediated killing of CLL B cells were unsuccessful. Therefore, it was surprising to discover that the dimers produced by the method of the present invention were capable of inducing apoptosis of B cells from CLL patients.

The anti-CD23 antibodies produced by the subject invention can be used for treatment of conditions including the following:

Allergic bronchopulmonary aspergillosis; Allergic rhinitis Autoimmune hemolytic anemia; Acanthosis nigricans; Allergic contact dermatitis; Addison's disease; Atopic dermatitis; Alopecia areata; Alopecia universalis; Amyloidosis; Anaphylactoid purpura; Anaphylactoid reaction; Aplastic anemia; Angioedema, hereditary; Angioedema, idiopathic; Ankylosing spondylitis; Arteritis, cranial; Arteritis, giant cell; Arteritis, Takayasu's; Arteritis, temporal; Asthma; Ataxia-telangiectasia; Autoimmune oophoritis; Autoimmune orchitis; Autoimmune polyendocrine failure; Behcet's disease; Berger's disease; Buerger's disease; bronchitis; Bullous pemphigus; Candidiasis, chronic mucocutaneous; Caplan's syndrome; Post-myocardial infarction syndrome; Post-pericardiotomy syndrome; Carditis; Celiac sprue; Chagas's disease; Chediak-Higashi syndrome; Churg-Strauss

herpetiformis; Dermatomyositis; Diabetes mellitus; Diamond-Blackfan syndrome; DiGeorge syndrome; Discoid lupus erythematosus; Eosinophilic fasciitis; Episcleritis; Drythema elevatum diutinum; Erythema marginatum; Erythema multiforme; Erythema nodosum; Familial Mediterranean fever; Felty's syndrome; Fibrosis pulmonary; Glomerulonephritis, anaphylactoid; Glomerulonephritis, autoimmune; Glomerulonephritis, post-streptococcal; Glomerulonephritis, post-transplantation; Glomerulopathy, membranous; Goodpasture's syndrome; Graft-vs.-host disease; Granulocytopenia, immune-mediated; Granuloma annulare; Granulomatosis, allergic; Granulomatous myositis; Grave's disease; Hashimoto's thyroiditis; Hemolytic disease of the newborn; Hemochromatosis, idiopathic; Henoch-Schoenlein purpura; Hepatitis, chronic active and chronic progressive; Histiocytosis X; Hypereosinophilic syndrome; Idiopathic thrombocytopenic purpura; Job's syndrome; Juvenile dermatomyositis; Juvenile rheumatoid arthritis (Juvenile chronic arthritis); Kawasaki's disease; Keratitis; Keratoconjunctivitis sicca; Landry-Guillain-Barre-Strohl syndrome; Leprosy, lepromatous; Loeffler's syndrome; lupus; Lyell's syndrome; Lyme disease; Lymphomatoid granulomatosis; Mastocytosis, systemic; Mixed connective tissue disease; Mononeuritis multiplex; [illegible] multiple sclerosis;

Myasthenia gravis; Mycosis fungoides; Necrotizing vasculitis, systemic; Nephrotic syndrome; Overlap syndrome; Panniculitis; Paroxysmal cold hemoglobinuria; Paroxysmal nocturnal hemoglobinuria; Pemphigoid; Pemphigus; Pemphigus erythematosus; Pemphigus foliaceus; Pemphigus vulgaris; Pigeon breeder's disease; Pneumonitis, hypersensitivity; Polyarteritis nodosa; Polymyalgia rheumatic; Polymyositis; Polyneuritis, idiopathic; Portuguese familial polyneuropathies; Pre-eclampsia/eclampsia; Primary biliary cirrhosis; Progressive systemic sclerosis (Scleroderma); Psoriasis; Psoriatic arthritis; Pulmonary alveolar proteinosis; Pulmonary fibrosis, Raynaud's phenomenon/syndrome; Reidel's thyroiditis; Reiter's syndrome, Relapsing polychrondritis; Rheumatic fever; Rheumatoid arthritis; Sarcoidosis; Scleritis; Sclerosing cholangitis; Serum sickness; Sezary syndrome; Sjogren's syndrome; Stevens-Johnson syndrome; Still's disease; Subacute sclerosing panencephalitis; Sympathetic ophthalmia; Systemic lupus erythematosus; Transplant rejection; Ulcerative colitis; Undifferentiated connective tissue disease; Urticaria, chronic; Urticaria, cold; Uveitis; Vitiligo; Weber-Christian disease; Wegener's granulomatosis; Wiskott-Aldrich syndrome.

Of these, the preferred indications treatable or presentable by administration

[illegible] inflammatory diseases and conditions.

The antibody molecules produced by the method of the present invention can be used in pharmaceutical compositions for any application wherein antibodies are therapeutically beneficial, e.g., the treatment of cancer and autoimmune disorders in mammals, especially humans. The genetically engineered antibodies of the present invention can be formulated according to known methods to prepare pharmaceutically useful compositions such as by admixture with a pharmaceutically acceptable carrier vehicle. Suitable vehicles and their formulation are described, for example, in Remington's Pharmaceutical Sciences (16th Ed., Osol, A. Ed., Mack Easton PA (1980)). To form a pharmaceutically acceptable compositions suitable for effective administration, such compositions will contain an effective amount of antibody, either alone, or with a suitable amount of carrier vehicle, e.g., a buffered saline solution.

The therapeutic compositions of the invention will be administered to an individual in therapeutically effective amounts. That is, in an amount sufficient to treat a particular condition, e.g., a cancer or an autoimmune disorder. The effective amount will vary according to the weight, sex, age and medical history of the individual. Other factors include the severity of the patient's condition, the mode

in dosages ranging from about 0.01 to about 2 picomoles/ml, more generally about 0.0001 to about 200 picomoles/ml.

The pharmaceutically prepared compositions may be provided to a patient by any means known in the art including oral, intranasal, subcutaneous, intramuscular, intravenous, intraarterial, parenteral, etc.

Having now generally described the invention, the following examples are offered by way of illustration only and are not intended to be limiting unless otherwise specified.

EXAMPLE 1

Production of Genetically Engineered C2B8/SH (NTB #:2012-85 and 2092/64)

a. Generation of C2B8/SH Anti-CD20 (Version 1) Cell Line:

It has been previously demonstrated by Shopes (*J. Immunol. 148(9)*:2918-2922 (1992), and Shopes et al, WO 91/19515, December 26, 1991) that "tail-to-tail" dimeric immunoglobulin (L_2H_2)$_2$ molecules can be induced through formation of a disulfide linkage between individual L_2H_2 immunoglobulin molecules. A similar approach was used by Caron et al. (*J. Exp. Med. 176*:1191-95 (1992)). Both groups artificially introduced a cysteine four amino acids from the carboxyl end of the

In an effort to create a dimeric anti-CD20 immunoglobulin, applicants similarly introduced a cysteine residue within the chimeric anti-CD20 antibody, C2B8. Figure 1 shows the nucleotide and predicted amino acid sequence of the murine anti-human CD20 light chain variable domain fused to the human kappa light chain constant domain. Figure 2 shows the nucleotide and predicted amino acid sequence of the murine anti-human CD20 heavy chain variable domain fused to the human gamma 1 heavy chain constant domain.

Through the use of conventional in vitro site directed mutagenesis, applicants effected a transversion mutation C to G within the plasmid DNA (Figure 3). This IDEC proprietary expression construct (Reff et al., U.S. Patent Appl. Serial No. 08/819,866, Filed March 14, 1997) encodes the anti-CD20 immunoglobulin light and heavy chains, as well as sequences necessary for homologous integration into a proprietary CHO cell line (Reff et al. IBID), followed by dominant selection with G418 and or methotrexate. The affect of this nucleotide mutation is to change the codon second base, thereby encoding a cysteine residue substituted for the normal serine residue at position 445 near the gamma 1 heavy chain carboxyl terminus (see Figure 2).

line designated 15C9 which was originally derived from CHO DG-44 (Urlaub et al.,

Som. Cell Mol. Gen. 12(6):555-566, 1986). Following selection with G418, a high level immunoglobulin producing clone, termed 3F9, was isolated. 3F9 produces and secretes into the cell growth medium, roughly 3.4 pg/cell/day of immunoglobulin. The ELISA assay of immunoglobulin productivity measures L_2H_2 immunoglobulin molecules irrespective of their monomeric, dimeric or oligomeric configuration. As evidenced by western blot analysis, the majority of the secreted immunoglobulin is monomeric (L_2H_2). However, a small percentage is in the dimeric and larger oligomeric forms.

The 3F9 cell line was then selected in 5 nM methotrexate. Growth in methotrexate can be used to artificially induce gene amplification (Alt et al., *J. Biol. Chem. 253*:1357-1370 (1978)) and expression of the plasmid encoded DHFR gene. Concomitantly, the linked immunoglobulin light and heavy chain genes will also be amplified resulting in increased immunoglobulin gene expression and higher immunoglobulin protein production. Through gene amplification, we were able to effectively induce an increase in total anti-CD20 production levels. Following selection, the clone designated 3F9-50B11 was identified. 3F9-50B11 produces roughly 6.3 pg cell/day of anti-CD20 protein.

C2B8/SH was purified from growth media (12L at 15 mg/L) using protein A (pA) column Chromatography. Sodium azide (0.01% final concentration) was added to the C2B8/SH antibody containing media and pH adjusted to 7.5 with 10N NaOH. The material was applied to a PBS washed pA affinity column (15 ml column, Bioprocess Ltd.) at approximately 3 ml/min. in a 4-8°C cold room, followed by washing with at least 5 column volumes PBS (100 ml). Antibody was eluted from the pA column with 100 ml Sodium Citrate (0.1 M, pH 3.5), and immediately neutralized to pH 7 with 1M Tris Base. C2B8/SH (pA purified) was dialyzed against PBS (1000 ml x 4 changes over 3 days), concentrated to approx. 10 mg/ml under Nitrogen (50 psi) in an Amicon stirred cell concentrator (MWCO 30,000), and filter (0.2 μm) sterilized. The pA purified C2B8/SH material was stored at 4°C. Protein concentration was determined spectrophotometrically: MAb (mg/ml) = [Absorbence at OD280] x [dilution factor] / 1.7.

c. <u>Characterization C2B8 Homodimer:</u>

C2B8/SH IgG (150 kDa) having a genetically engineered thiol group in the antibody heavy chain is able to form a 300 kDa IgG/IgG homodimer through intermolecular disulfide linkage. The amount of homodimer formed was

[illegible] was performed

using a Beckman 126 HPLC system operating isocratically at a flow rate of 1.0 ml/min., with a mobile phase consisting of 100 mM sodium phosphate, 150 mM sodium chloride, pH 6.8. The separation was performed at room temperature using a 7.8 x 300 mm BioSil SEC 250-5 column (Bio-Rad Catalog #125-0062) monitored by Absorbence at 280 nm. Molecular weights were approximated by comparison to an external Bio-Rad Gel Filtration Standard (Bio-Rad Catalog #151-1901).

Non-Reducing SDS/PAGE gels of CHO secreted C2B8/SH (Figure 5, Lane 1) showed a major protein band at 150kDa (IgG) and HPLC analysis of several preparation showed ≤ 6% IgG/IgG homodimer (300kDa) in MAb containing growth medium. After pA purification and concentration, three major protein bands were observed (Figure 5, Lane 2). Molecular weight determination by HPLC showed the three protein peaks at 150 kDa (80.3%), 300 kDa (14.9%) and >450 kDa (4.8%). HPLC results from several C2B8/SH pA purifications showed homodimer ranges from 12.5-17.9% (Figure 7, 8) which was comparable to the amount of MAb homodimers synthesized by Ghetie et al. (*PNAS, USA 49*:7509-7514 (1997)), who used hetero-bifunctional cross-linking agents to chemically couple the IgG monomers.

94:75-81 (1979)). Despite the observation that >80% of the C2B8/SH remained monomer after dimerization, very little reactive thiol was detected (< 0.2 SH groups per MAb), indicating that the genetically introduced thiol on the IgG heavy chain was blocked, most likely through intermolecular disulfide bridging

EXAMPLE 2

(Ntb #:1966/84): Selective Reduction of C2B8/SH and Preparation of C2B8, Disulfide Linked, Homodimer (Figure 4.1)

To increase the percentage of dimer in the C2B8/SH preparation, pA purified material was partially reduced with a 2-fold molar excess of dithiothreitol (DTT), concentrated, and allowed to form antibody dimers in PBS under normal atmospheric conditions. MAbs partially reduced using DTT for use in preparing affinity columns (Goldenberg et al., *Bioconj. Chem. 2*:275-280 (1991)) or for immunoconjugate preparations (Siegall et al., *Bioconj. Chem. 3*:302-307 (1992), Willner et al., *Bioconj. Chem. 4*:521-527 (1993)), have been shown to maintain their molecular integrity (150 kDa), and antigen binding capacity.

a. Selective Reduction C2B8/SH:

This method used DTT to partially reduce either the intra or inter molecular disulfide bond and allow IgG-IgG dimers to reform more efficiently. 0.0145 mg of pH 7.4 (cmfPBS.) was added to 21.8 mg of pA purified MAb C2B8/SH in cmfPBS

containing 3.5 mM Na_2-EDTA, to give a final ratio of 2.0 moles DTT per mole of MAb. The reaction was immediately degassed and incubated under nitrogen for three hours at 37°C. The MAb was purified from unreacted material using Sephadex G-25 column chromatography (PD-10 columns, Pharmacia Fine Chemicals) that was equilibrated with PBS. The MAb containing fraction was collected according to manufacturers instructions in a final volume of 3.0 ml equilibration buffer (PBS). The selectively reduced C2B8/SH was further incubated for two hours at room temperature in air. The reaction was terminated by the addition of 0.1 ml (100mM) cysteine, and concentrated using an Ultrafuge™ concentrator with a 30,00 MWCO. Protein concentration was determined by absorbance at 280nm (1 mg/ml= 1.7AU).

b. <u>Characterization C2B8 (-s-s-) Homodimer:</u>

The material was stored at 4°C until analysis using SDS/PAGE (Figure 5, lane 4) and analytical HPLC (Figure 7, Table I). Disulfide linked homodimer, increased from 17.5% in the starting material to 39.4% after selective reduction and dimerization. Repeat synthesis using this method showed dimers ranging from 39.4% of the population to 51% of the starting material.

[illegible] preparative HPLC [illegible] Preparative SE

HPLC was performed using a Beckman 126 HPLC system operating isocratically at a flow rate of 4.0 ml/min. with a mobile phase consisting of 100 mM sodium phosphate, 150 mM sodium chloride, pH 6.8. The separation was performed at room temperature using a 21.5 x 75 mm TosoHaas TSK-Gel SW guard column attached to a 21.5 x 300 mm TosoHaas TSK-Gel G3000-SW column. Fractions were collected manually by monitoring the computer trace of Absorbence at 280 nm in real time. In general, homodimers were >95% pure after HPLC purification.

EXAMPLE 3

(Ntb #:1966/78): Preparation of C2B8, Thioether Linked, Homodimer (Figure 4.2)

a. Selective Reduction C2B8:

5.45 mg of pA purified C2B8/SH (7.27×10^{-5} M) in 0.5 ml cmfPBS/EDTA was reduced with a 2 fold molar excess of DTT for three hours at 37°C using conditions described in example 2. The selectively reduced MAb was applied to a PD-10 column, equilibrated with deoxygenated normal saline containing sodium citrate (10mM) and EDTA (1mM) buffered to pH 6.3 using hydrochloric acid (Saline/Citrate buffer). The first antibody containing peak, in 3.0 ml equilibration buffer (Saline Citrate buffer), was collected following manufacturer instructions.

The thiol concentration (SH content) estimated using Ellmans reagent was found to average approximately 2 moles of free thiol for each mole DTT-reduced C2B8/SH. Molecular integrity was confirmed with this method using SDS non-reducing PAGE.

b. <u>Homodimer (-s-) Reaction:</u>

Bismaleimidohexane (BMH, Pierce Chemical Co. Product #:22319) was diluted to 10mM in DMF and added to the selectively reduced C2B8/SH to give a final molar ratio of 2.5 moles BMH per mole MAb. The mixture was rotated for 2.5 hours at room temperature in a N_2 atmosphere. The reaction was terminated by the addition of 0.1 ml Cysteine (100mM in PBS) and stored at 4°C (normal atmosphere) until analysis and purification using HPLC.

The mixture was analyzed using the analytical HPLC method described in example 2. The fraction (300 kDa) containing the thioether linked (-s-) C2B8 homodimer represented 28% of the total protein collected (Figure 7 and Table 1). Preparative HPLC (as described in example 2) was used to purify the (-s-) homodimer from the unpurified mixture with purity typically >95%, as determined by SDS-PAGE (non-reducing) gels and analytical HPLC (results not shown).

[illegible] approximately slow [illegible] kDa [illegible]

chain), 55 kDa (H-chain) and 110 kDa (H-H dimer) (Figure 6, Lane 7). In contrast, disulfide linked homodimer or monomer Ab showed the 2 expected protein bands at 22 and 55 kDa.

EXAMPLE 4

(Ntb #;1266/85): Preparation of C2B8, Thioether Linked, p5E8 Heterodimer (Figure 4.3)

a. Selective Reduction C2B8:

Purified C2B8/SH, 10.9 mg in 1.0 ml cmfPBS ($7.27x10^{-5}$M,), was reduced using a 2 fold molar excess of DTT (three hours at 37°C, N_2 atm.), using conditions described in example 2, and purified using PD-10 columns equilibrated with Saline/Citrate buffer. The molar ratio of thiol to MAb, determined using Ellmans reagent, as described in example 3, was 1.2. Reduced C2B8/SH was immediately mixed with MAb p5E8 (anti-CD23) that was previously modified with Succinimidyl 4-(p-maleimidophenyl)-butyrate (SMPB, Pierce Chemical Co., Product #22315).

b. SMPB Modified p5E8:

MAb p5E8 ($4.5x\ 10^{-5}$ M in PBS) was functionalized by addition of a 6 fold molar excess of SMPB (10mM in DMF) and rotating the mixture for [illegible]

PD-10 columns equilibrated with Saline/Citrate buffer. Protein concentration of the SMPB functionalized MAb was determined spectrophotometrically:

MAb (mg/ml)=[Absorbence 280] x [dilution factor] / 1.5

c. Heterodimer Formation:

Heterodimer (anti-CD20/anti-CD23) was prepared by mixing 1.5 mole equivalents of SMPB containing p5E8 (11.37 mg) with 1 mole equivalent freshly reduced C2B8/SH (8.0 mg) for one hour at room temperature in a N_2 atm. Heterodimer was analyzed and purified using HPLC, as described in example 2. Figure 8 and Table 2 show HPLC chromatograms of unpurified and purified heterodimer compared to starting material. Purity of the 300 kDa heterodimer was >95%, as determined by analytical HPLC (Table 2) and non-reducing and reducing SDS-PAGE gels Figure 6. Reducing SDS/PAGE (Figure 6, lane 6) also showed three major protein bands after reduction, including a non-reducible 110 kDa band, consistent with the formation of thioether linked H-H dimer.

EXAMPLE 5

Binding Activity of C2B8 Homodimer and C2B8/p5E8 Heterodimer

Binding of monomer and dimerized antibody to various cells was evaluated

Serum/0.1 % Sodium Azide, PBS/FCS buffer) were incubated for one hour on ice with 0.1 ml of 5 fold serially diluted antibody. Cells were twice washed by centrifugation (200x g) using 2 ml PBS/wash and suspended in 0.2 ml FITC conjugated *Goat* $(Fab')_2$ anti-human IgG (Jackson Immunoresearch #30869, 5 μg/ml in PBS/FCS buffer). After 30-min. incubation on ice, cells were again washed in PBS and suspended in 0.2 ml 0.5% freshly diluted formaldehyde, capped and stored at 4°C until analysis. The amount of cell bound antibody was determined by flow cytometry (FACScan, Becton-Dickenson, Mountain View, CA).

a. C2B8 Homodimer:

Figure 9 compares the binding of MAbs: C2B8 (disulfide linked) homodimer, C2B8, and RF-2 on the CD20+/CD23+ positive cell lines, SKW and SB. RF-2 was used as an isotype matched non-binding antibody control. Similar binding curves for both the C2B8 monomer and dimer was obtained on both cell lines, suggesting similar binding activity for the CD20 antigen.

The binding affinity of the C2B8 homodimer and monomer for the CD20 antigen was compared using a competitive binding assay (Figure 10). SKW cells were first incubated for 30 minutes on ice with various amounts of 5-fold serially

[illegible] Figure [illegible] evaluated the

amount of C2B8 binding. Previous experiments had demonstrated no reactivity of the FITC anti-human IgG for the murine 2B8 antibody. The concentration of C2B8 that gave 50% inhibition of 2B8 antibody binding was 9.8 μg/ml, and 10.4 μg/ml for the homodimer. Data of both Figures 9 and 10, therefore, indicate no significant effect on binding affinity for the CD20 antigen as a result of dimerization to a 300 kDa species. Direct staining and FCM analysis, as described in Figure 9, using thioether linked C2B8 homodimer was similar to results obtained using the disulfide linked dimer (not shown).

b. <u>C2B8/p5E8 Heterodimer:</u>

Binding of C2B8/p5E8 Heterodimer, C2B8 and p5E8 on SKW (CD20+/CD23+) and DHL4 (CD20+/CD23-) cells is shown on Figure 11. Similar binding curves comparing monomer to heterodimer were obtained on both cell lines, including CD23 antigen negative DHL-4 cells. The data strongly suggested that the heterodimer, like the anti-CD20 homodimers, retained full functional binding for the CD20 antigen.

To determine heterodimer binding activity for the CD23 antigen, SKW cells (1×10^6 cells PBS FCS buffer) were first incubated with a saturating amount

[illegible] direct staining preparations of figure 12. Murine 2B8 completely

inhibited binding of both monomer and dimerized C2B8 antibody, but did not effect the binding of either p5E8 or of the Heterodimer. The data suggests that the heterodimer also retained full functional binding activity for the CD23 antigen after dimerization with C2B8.

EXAMPLE 6

Anti-tumor Activity of C2B8 Homodimer in Murine Animal Models

The Daudi human lymphoma tumor line was established in BALB/c nu/nu mice from tissue culture and maintained as a tumor xenograft via sc. inoculation of tumor Brie. Caliper measurements in two perpendicular directions at weekly intervals measured tumor size. Tumor volume was estimated from size measurements by the formula: Tumor Volume (mm^3) = Length x $(Width)^2$ / 2

MAb treatments were administered i.p. on various schedules indicated for each experiment. Antibody was diluted in PBS and administered i.p. as mg per mouse with 8 animals in each group. Control groups remained untreated. Data is reported as median tumor volume for control or treated animal groups. A complete regression was defined as a failure to detect tumor for at least two measurements (> 2 weeks).

[illegible] compares anti-tumor activity of low-dose C2B8

μg/mouse) C2B8 homodimer (schedule: every 5 days x 3 injections, Q5dx3) to the activity of dose and schedule optimized C2B8 monomer (1 mg/mouse, Q5dx2). MAb treatment was initiated on established tumors, 50-150 mm^3 at start of treatment. At this dose and schedule, the C2B8 homodimer showed tumor growth inhibition comparable to dose optimized C2B8. By day 65, 50% of the animals treated with 200 ug x3 doses of C2B8 homodimer showed complete tumor regression. Animals receiving 1 mg x 2 doses of C2B8 had 37.5% complete regressions.

Figure 14 compares the activity at matching schedules (Q5dx3) of 200 μg/mouse C2B8 monomer or homodimer on established tumors 150-250 mm^3 in size. Tumor growth of the C2B8 homodimer treated mice was inhibited to a greater extent than a comparable amount of the C2B8 monomer. At this dose (0.2 mg/mouse), 62.5% of the homodimer treated mice had completely regressed tumors, while 25% of monomer treated mice showed complete tumor regression.

EXAMPLE 7

Apoptotic Activity of C2B8 Disulfide Linked Homodimer on B Cell Lymphoma Cells

The ability of homodimers to induce apoptosis of CD20$^+$ B cell lymphoma

CD23$^+$) cells ($1x10^6$ cells/ml) at log-phase of growth. The cells were propagated in RPMI 1640 (Irvine Scientific) plus 5% Fetal Bovine Serum (FBS) with 2 mM L-Glutamine (Irvine Scientific) and 100 U/ml of Penicillin-Streptomycin (Irvine Scientific) at 37°C in 5% CO_2) incubator. As controls, cultures were incubated with either C2B8 monomer or a irrelevant isotype matched antibody control, RF2. After 72 hours of incubation, cells were harvested by centrifugation at 350 x g for 5 minutes and fixed with 70% (v/v) ethanol (ice-cold) for 30 minutes. Fixed cells were analyzed for apoptosis by a flow cytometry based TUNEL assay using APO-BRDU™ Kit as per manufacturer's instructions (Pharmingen). The treatment of DHL-4 and SKW cells by C2B8 homodimer showed evidence of apoptotic death of cells dependent on the dose of antibody used (Figure 15 and Table III). In contrast, treatment of cells with same concentrations of C2B8 monomer or the control antibody, RF2 showed no evidence of apoptosis. In addition, with Ramos cells (CD20$^+$ Burkitt's lymphoma cell line) that are susceptible to higher degree of spontaneous apoptosis in culture, the addition of homodimers to these culture resulted in enhaneed apoptosis (Figure 15).

EXAMPLE 8

The ability of heterodimers to induce apoptosis of CD20$^+$ B cell lymphoma cells was determined by TUNEL assay. B lymphoma cells were grown and evaluated, as described in Example 7. Briefly, varying concentrations of heterodimer were added to DHL-4 and SKW cells at log-phase of growth and tested for apoptosis induction as described above in Example 7. As controls, cultures were incubated with C2B8 monomer, p5E8 monomer and an irrelevant antibody control, RF2. Figure 16 and Table III show the induction of apoptosis in DHL-4 and SKW cells by C2B8 heterodimer in a dose-dependent manner. In cells cultured with C2B8 and p5E8 monomers or the control antibody RF2, no evidence of apoptosis was observed.

EXAMPLE 9

C2B8 Homodimers mediated complement dependent cytotoxicity of normal B cells

The ability of C2B8 homodimers to mediate killing of peripheral blood B cells by complement dependent cytotoxic (CDC) mechanism was demonstrated using a modified flow cytometry based assay. Peripheral blood mononuclear cells (PBMC) were isolated from blood of healthy human donors by Ficoll-Hypaque gradient centrifugation. Viability was determined by Trypan blue dye exclusion

and washed with 2 ml of HBSS by centrifugation and aspiration of supernatant to remove unbound antibodies. The cell pellet was re-suspended with 100 µl rabbit complement (ICN/Cappel Cat. #55866) at different dilutions and incubated for 60 minutes at 37°C. After incubation, 10µl of anti-CD19-FITC antibody (Pharmingen) was added. Cells were incubated on ice for 30 minutes, followed by addition of 50 µl (20µg/ml) of Propidium iodide (PI; Boehringer Mannheim). Fifteen minutes later, 400µl of HBSS was added to all tubes and the cells were immediately analyzed by FACScan (Becton-Dickinson).

Data was analyzed using the WinList software package, as described by the manufacturer (Variety Software House). Purity of the lymphocyte preparation used for the assay was found to be greater than 95% as determined by the Leucogate (CD45 positive cells). The $CD19^+$ cell (B cell lineage) population of the total lymphocyte population ($CD45^+$) was gated for further analysis. The percentage of $CD19^+$ cells incorporating PI represented the dead or dying cell population and was determined using the WinList Software. Data in Table I show that the C2B8 homodimer is effective in mediating CDC of peripheral $CD19^+$ B cells. Cells incubated with complement alone at 1:10 and 1:20 dilutions (Table IV) had a 20%

incubated with C2B8 homodimer (70% increase over control). Control cells incubated without complement showed less than 10% cytotoxicity (data not shown).

Table I. Complement-Dependent Cytotoxicity of C2B8 Dimers on CD19$^+$ B Cells

Antibody[a]	% Cytotoxicity[b] Complement dilution 1:10	1:20
C2B8 dimer	34.19	41.29
C2B8 monomer	28.89	23.86
No antibody control	20.10	20.28

[a] Antibody was tested at the optimum concentration of 2 μg/ml, as determined from a previous experiment.

[b] % Cytotoxicity was determined as the percentage of CD19$^+$ cells that showed uptake of propidium iodide stain.

EXAMPLE 10

Growth Inhibition of B Cell Lymphomas by C2B8 Homo and Hetero Dimers

The ability of homodimers and heterodimers to directly inhibit the growth of B lymphoma cell lines SKW and SB was determined by a proliferation inhibition assay. Briefly, varying concentrations of C2B8, p5E8, C2B8 homodimer and C2B8-p5E8 were added to $5x10^5$ in 96-well flat bottom plates in 200μl of growth

(Biosource International, Cat. DAL 1100) was added to each well. Following incubation, plates were cooled to room temperature for 10 minutes on a shaker and the intracellular reduction of the dye was determined and fluorescence was read using a 96-well fluorometer with excitation at 530nm and emission at 590nm.

The results are expressed as relative fluorescence units (RFU). The percentage growth inhibition was calculated as: [1 -(Average RFU of Test sample ÷ Average RFU of no antibody control)] x 100%. As indicated in the Figure 17, C2B8 homodimer and the heterodimers showed inhibition of both SKW and SB cell growth in vitro in a dose-dependent manner. Consistent with our previous findings, the C2B8 and p5E8 monomers did not inhibit growth of SKW and SB cells. In contrast, both C2B8 (-s-s-) and C2B8-p5E8 (-s) showed dose dependent inhibition of cell growth. IC50 values for homodimer were 0.625 μg/ml for SKW and for SB cells, while IC 50 values for the heterodimers raised from 0.625 μg/ml to 1.41 μg ml.

EXAMPLE 11

Growth Inhibition of B Cell Lymphoma by Cross-linking of C2B8 Monomers

The ability to enhance the biological activity by hyper cross-linking of

[illegible] SB cells in RPMI-1640

growth medium containing 10% FCS was added to each well of 96-well U-bottom plate and incubated with increasing concentrations of C2B8. After 1 hour of incubation at 37°C, 50 µl of murine monoclonal anti-human IgGI antibody (Sigma Chemical Co.) at 10 µg/ml of final concentration was added to each well and incubated for an additional 72 hours. During the last 18 hours incubation, cultures were pulsed with 1µ Ci per well of [^{3}H]-thymidine. Cells were washed, harvested and cell-associated radioactivity measured using an automated liquid scintillation counter.

A representation of the data from the cell proliferation experiment is shown in Figure 18, which indicates that hyper cross-linking of C2B8 on the surface of B cell lymphoma using a secondary antibody showed a clear dose dependent inhibition of cell proliferation, which was not observed when CD20$^+$ B cells were incubated with monomeric C2B8. Antibodies tested under similar conditions on CD20 HSB cells showed no effect, indicating that the observed effect was mediated via the CD20 molecule on the surface of B cell lymphomas. In addition, cross-linking of C2B8 by direct coating of culture wells without a secondary antibody prior to the addition of cells also resulted in inhibition of cell growth, further

EXAMPLE 12

Apoptotic Activity of C2B8 Disulfide Linked Homodimer on PBMC Isolated from a CLL Patient

The ability of C2B8 homodimer to induce apoptosis using CD20$^+$ B cells from human patients diagnosed with chronic lymphocytic leukemia (CLL) was also determined by TUNEL assay. Disulfide linked homodimer was compared to monomer for apoptosis induction on lymphocytes isolated from a donor diagnosed with CLL. The PBMC were cultured in RPMI 1640 medium supplemented with 2% donor plasma, plus 2mM L-Glutamine and 100 U/ml of Penicillin-Streptomycin. As controls, cultures were incubated with C2B8 monomer and the non-binding MAb RF2. After 120 hours of incubation, cells were harvested and fixed with 70% (v/v) ethanol and analyzed for apoptosis by TUNEL assay, as descried earlier (Example 7). The treatment of leukemic cells by C2B8 homodimer resulted in approximately 20% increased cell death by apoptosis, compared to cells that were with the same concentrations of C2B8 monomer or the control antibody, RF2 (Table II). Overall, a high level of spontaneous apoptotic cell death was observed with CLL-B cell, which may be the result of the suboptimal culture conditions used in these studies.

Table II: Induction of Apoptosis by C2B8 Homodimer of CD 19$^+$/CD20$^+$ B Cells from a CLL Patient

Clinical Sample	Treatment	Apoptosis[a]		
		10 μg/ml	2.5 μg/ml	0.625 μg/ml
CSK#1	C2B8-C2B8	84%	83%	65%
	C2B8	64%	65%	63%
	RF2	62%	67%	60%

[a] Apoptosis was determined by Tunel assay, as described under example 7. Degree of apoptosis was expressed as % apoptosis by sample divided by % apoptosis of controls. Flow cytometric analysis was performed on Becton-Dickinson FACScan using a FACScan Research Software package and the final data analysis was performed using the WinList Software package (Variety Software House). Percentage of cells positive for apoptosis was determined as the percentage of gated cells that were positive above the background, autofluorescence.